

# Effect of transport enrichment media, transport time, and growth media on the detection of *Campylobacter fetus* subsp. *venerealis*

Holly Monke<sup>1</sup>  
Senior Honors Research Project

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## Abstract

Bovine genital campylobacteriosis is a contagious venereal disease of cattle caused by *Campylobacter fetus* subsp. *venerealis*. Semen collected from a bull infected with *C. fetus* subsp. *venerealis* can be contaminated and the bacteria transmitted to thousands of cows by artificial insemination. Reliable diagnostic procedures are required to accurately test semen donor bulls and to prevent seminal transmission of disease.

The purpose of this study was to determine a combination of microbiological growth conditions such as transport enrichment media (TEM), transport time, TEM incubation, and growth media that best yields *C. fetus* subsp. *venerealis* while inhibiting contaminants. Transport enrichment medias evaluated include Weybridge, Cary and Blair, and 0.85% saline solution. Each TEM was inoculated with preputial smegma spiked with *C. fetus* subsp. *venerealis* and transported for 4 or 24 hours before being inoculated onto growth media with and without overnight incubation at 37 C. *C. fetus* subsp. *venerealis* and contamination growths were evaluated on a scale of 0-4. Median scores of *C. fetus* subsp. *venerealis* and microbial contamination were compared within TEM, transport time, overnight incubation, and growth media groups using the Mann-Whitney U test and Kruskal-Wallis test. The proportion of samples with any *C. fetus* subsp. *venerealis* growth or microbial contamination within each group was compared using the chi square test.

The results suggest that *C. fetus* subsp. *venerealis* growth was significantly influenced by three of the four criteria. Weybridge TEM more effectively promoted growth than either Cary and Blair TEM or 0.85% saline solution ( $P<0.0001$ ). Transport time of 4 hours rather than 24 hours was superior ( $P<0.0001$ ). Benefits were associated with avoiding overnight TEM incubation at 37 C ( $P=0.0002$ ). Significant differences were not identified for growth media;

however, Skirrow's Campylobacter Agar yielded slightly better growth than either blood agar or Greenbriar Plus Agar.

Contaminant growth was also significantly influenced by three of the four variables. Differences associated with TEM indicated Weybridge TEM inhibited contaminant growth more effectively than either Cary and Blair TEM or 0.85% saline solution ( $P < 0.0001$ ). Transport times of 4 and 24 hours did not significantly influence contaminant growth. Abstaining from overnight incubation of TEM was preferred for reduction of contaminant growth ( $P = 0.0032$ ). Skirrow's Agar was preferred to both blood agar and Greenbriar Plus Agar ( $P < 0.0001$ ).

These results suggest that the detection of *C. fetus* subsp. *venerealis* is enhanced when preputial smegma samples arrive at the diagnostic laboratory within 4 hours of collection using Weybridge TEM followed by direct inoculation onto Skirrow's Agar the day samples arrive. Adherence to these guidelines will facilitate accurate diagnosis of bovine genital campylobacteriosis in bulls, thereby reducing the potential for seminal transmission of *C. fetus* subsp. *venerealis* and subsequent occurrence of infertility, early embryonic death, and abortion.

## Introduction

Bovine genital campylobacteriosis is caused by the gram-negative, microaerophilic, motile, spiral-shaped rod bacterium *Campylobacter fetus* subsp. *venerealis* that is harbored in the proximal preputial cavity of bulls and in the genital tract of cows (Fig. 1).<sup>2</sup> Transmission of the bacterium occurs mechanically between cows and bulls during coitus, by contaminated semen used for artificial insemination, or between bulls through contact with either contaminated bedding or contact with contaminated semen-collecting equipment.<sup>1</sup>

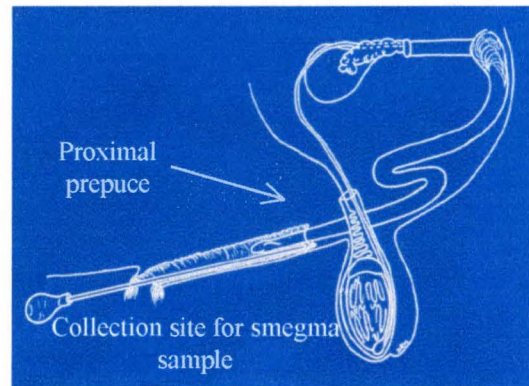
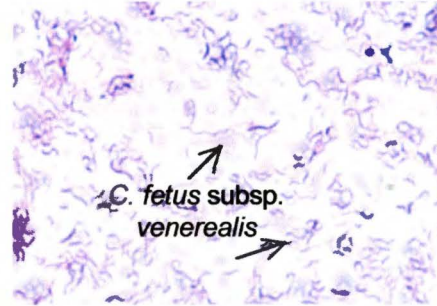
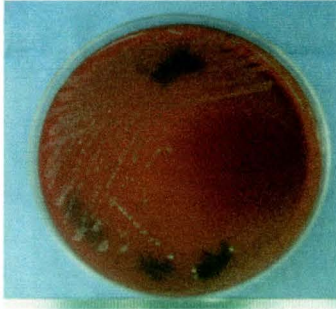


Figure 1. *C. fetus* subsp. *venerealis* appears as non-hemolytic, grayish-white convex colonies. The S-shaped morphology is apparent when counter-stained with carbol fuchsin (1000x oil immersion). Smegma samples are collected from a bull's preputial cavity. (Schematic from Monke, 1998)

Campylobacteriosis in the cow or heifer causes irregular estrous cycles, uterine infections that prevent conception, and early embryonic death.<sup>5,7</sup> Most cows recover from infection and begin estrous cycles after several months, but the herd experiences a decreased pregnancy rate and a prolonged calving season.<sup>7</sup> Subsequently, reproductive efficiency is decreased and if carriers are not identified and management actions taken, the disease can persist in the herd for years.

Disease control may be assisted by artificially inseminating cows with semen collected from bulls diagnosed free of *C. fetus* subsp. *venerealis* infection. In addition, international semen export requirements mandate that individual bulls or the entire bull herd be tested negative for *C. fetus* subsp. *venerealis*.<sup>12,13</sup> It is important, therefore, that the diagnostic test for *C. fetus* subsp. *venerealis* be able to accurately detect and identify infected bulls.

Preputial samples require careful handling because the bacterium has limited viability outside the host animal due to toxic effects of prolonged exposure to atmospheric levels of oxygen.<sup>3</sup> Furthermore, faster growing, non-pathogenic microorganisms ubiquitously present in the sample, such as *Pseudomonas* spp. and *Proteus* spp., may overgrow and contaminate the culture media, thus reducing the ability to identify *C. fetus* subsp. *venerealis*.<sup>3,10</sup> To minimize these problems and enhance bacterial viability, specimens are inoculated in a transport enrichment medium (TEM), transported to the diagnostic laboratory as soon as possible, cultured on selective growth media, and microaerophilically incubated.<sup>4,8,9</sup>

Each variable used in this study is currently employed in diagnostic procedures for culturing *C. fetus* subsp. *venerealis*. However, the optimal combination of TEM, growth media, transit time, and post-transit incubation that results in satisfactory recovery of *C. fetus* subsp. *venerealis* has not been determined.

## **Objectives**

The purpose is to determine the combination of TEM, transit time, laboratory processing procedure, and growth media that best maximizes growth of *C. fetus* subsp. *venerealis* while inhibiting growth of microbial contaminants.

## **Materials and methods**

### **Transport and culture media**

Cary and Blair transport media was prepared according to manufacturer's directions.<sup>a</sup> Prepared saline solution and Weybridge transport media were also used.<sup>b</sup> Blood agar contained Tryptic Soy Agar and 5% sterile defibrinated sheep blood.<sup>c</sup> Greenbriar Plus contained Eugon Agar, 5% sterile defibrinated sheep blood, albamycin, bacitracin, polymixin B, and cycloheximide.<sup>d</sup> Skirrow's media contained Bacto Campylobacter Agar Base, Bacto Campylobacter Antimicrobial Supplement S, and sterile defibrinated sheep blood.<sup>e,6</sup>

### **Preputial sample collection**

Preputial samples were collected on several occasions from 6 adult semen-donor bulls previously diagnosed negative for *C. fetus* subsp. *venerealis* and residing in a herd tested negative for *C. fetus* subsp. *venerealis*.<sup>f</sup> A disposable plastic pipette fitted with a sterile rubber bulb and containing 4 ml of 0.85% sterile saline solution was inserted into the prepuce. Smegma from the region near the preputial fornix was collected using a combination of scraping and aspirating.<sup>11</sup> Samples were placed into sterile 13x100mm glass tubes fitted with metal closures for transport to the diagnostic laboratory at 25 C within one hour. Upon arrival at the laboratory,

300µl of the sample were inoculated onto blood agar, Greenbriar Plus Agar, and Skirrow's Campylobacter Media to confirm the bulls' negative status.

### **Inoculation of preputial material**

Confluent colonies of *C. fetus* subsp. *venerealis* ATCC strain 19438 were propagated on blood agar plates and microaerophilically incubated for 48 hours at 37 C. Smegma was pooled into 20ml mixtures in 50ml plastic centrifuge tubes and spiked with *C. fetus* subsp. *venerealis*. Colonies were removed from blood agar using sterile cotton tipped swabs dipped in sterile 0.85% saline and swiped across the media. The swabs were swirled into the pooled preputial smegma samples to inoculate them with *C. fetus* subsp. *venerealis* colonies.

Serial dilutions of the spiked sample were used to determine the concentration of *C. fetus* subsp. *venerealis* present. Spiked smegma was diluted to mixtures of  $1 \times 10^{-1}$  through  $1 \times 10^{-5}$  colony forming units (cfu)/ml and 300µl of each dilution was spread with a sterile glass hockey stick onto blood agar plates and microaerophilically incubated for 48 hours at 37 C. Enumeration plates with 30-300 cfu's were used to calculate that spiked samples contained between  $6.9 \times 10^5$  to  $6.1 \times 10^6$  organisms/ml.

### **Inocula preparation**

*Experiment 1.* Three TEM (Cary and Blair, 0.85% saline solution, and Weybridge TEM) were inoculated with smegma spiked with *C. fetus* subsp. *venerealis* (Fig. 2). Cary and Blair transport media was inoculated by inserting a sterile cotton-tipped swab into the spiked smegma to thoroughly wet the cotton, placing the swab into a 13x100mm glass tube containing the media, and leaving the swab in the media to be covered with a stainless steel closure. One milliliter of spiked smegma was added to 9ml of 0.85% sterile saline. Weybridge media was inoculated with 300µl of spiked smegma and gently mixed.

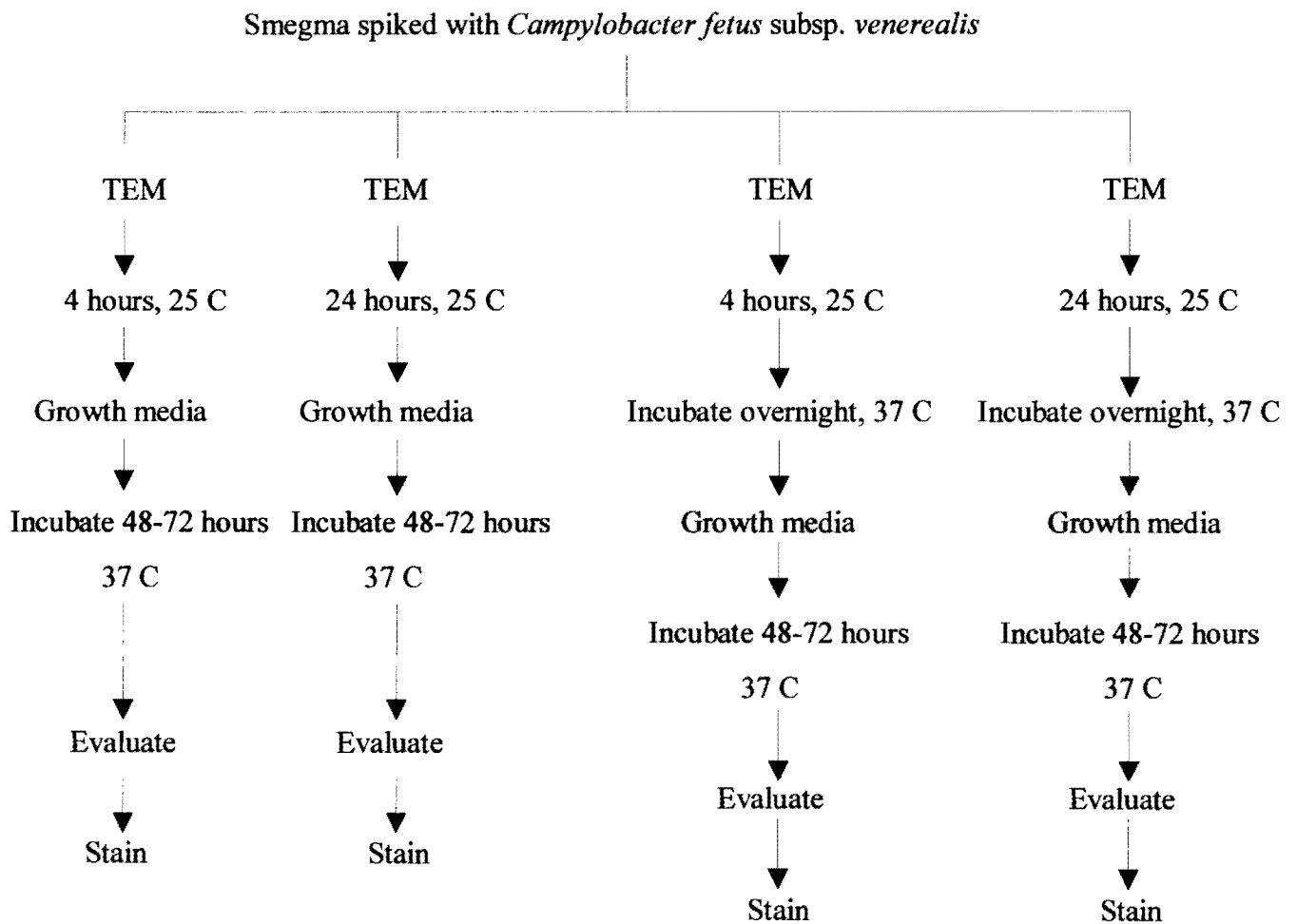


Figure 2. Sample processing procedure.



The three TEM were divided into two groups. These were held in the laboratory at either 25 C for 4 hours or 25 C for 24 hours to represent the times a sample would typically be in transit from collection site to the diagnostic laboratory.

Three growth media (blood agar, Greenbriar Plus, and Skirrow's Campylobacter Media) were inoculated with each TEM and transport time combination. The swab in the Cary and Blair media was swirled in 300µl of 0.85% sterile saline in 10x75mm sterile glass tubes to remove the colonies and allow uniform distribution of colonies. The 300µl of saline was spread onto each growth media using a sterile glass hockey stick. Each growth media was inoculated with 0.85% saline solution and Weybridge TEM by spreading 300µl of each solution onto each media using a sterile glass hockey stick. Growth media were microaerophilically incubated at 37 C for 48-72 hours.

*Experiment 2.* Transport enrichment media in experiment 2 were inoculated and remained at 25 C for 4 and 24 hours as were those in experiment 1. Following the pseudo-transport interval, TEM were aerobically incubated at 37 C overnight. Growth media were inoculated and incubated as in experiment 1.

### **Culture analysis**

Cultures were visually examined and evaluated for growth of *C. fetus* subsp. *venerealis* on a scale of 0-4 according to the number of colonies recovered. Inhibition of contaminant growth was also visually evaluated and ranked on a scale of 0-4 according to the percentage of growth media covered by microbial contaminant growth.

Diagnosis of *C. fetus* subsp. *venerealis* was confirmed by microscopic examination of colonies stained with crystal violet followed by iodine, decolorized with alcohol, and counterstained with carbol fuchsin then viewed at 1000x oil immersion.

## Statistical analysis

Median scores of *C. fetus* subsp. *venerealis* and microbial contamination were compared within TEM and growth media groups using the Mann-Whitney U test. Median scores were compared within transport time and overnight incubation groups using the Kruskal-Wallis test. The proportion of samples with any *C. fetus* subsp. *venerealis* growth or contamination within each of the four groups was compared using the chi square test.

## Results

Visual evaluation of samples suggests that microbial contaminants may adversely affect the detection of *C. fetus* subsp. *venerealis*. Fast-growing, large, or swarming contaminants such as *Pseudomonas*, *Proteus*, and mold reduce the growth media area available for *C. fetus* subsp. *venerealis* to be detected (Fig. 3).

The frequency of *C. fetus* subsp. *venerealis* growth in each score category indicates the efficacy of growth promotion (Table 1). Likewise, frequency of contaminant growth in each score category denotes the extent of overgrowth (Table 2). Analysis of the microbial growth associated with the variables of TEM, transit time, incubation, and growth media indicates the relative magnitude of *C. fetus* subsp. *venerealis* and contaminant growth. Variables that facilitate *C. fetus* subsp. *venerealis* culture growth are compared to variables that inhibit contamination in Figure 4.

Analysis of median score results using the Mann-Whitney U test and Kruskal-Wallis test suggest that *C. fetus* subsp. *venerealis* growth is significantly influenced by three of the four criteria. Weybridge TEM more effectively promotes growth than either Cary and Blair TEM or

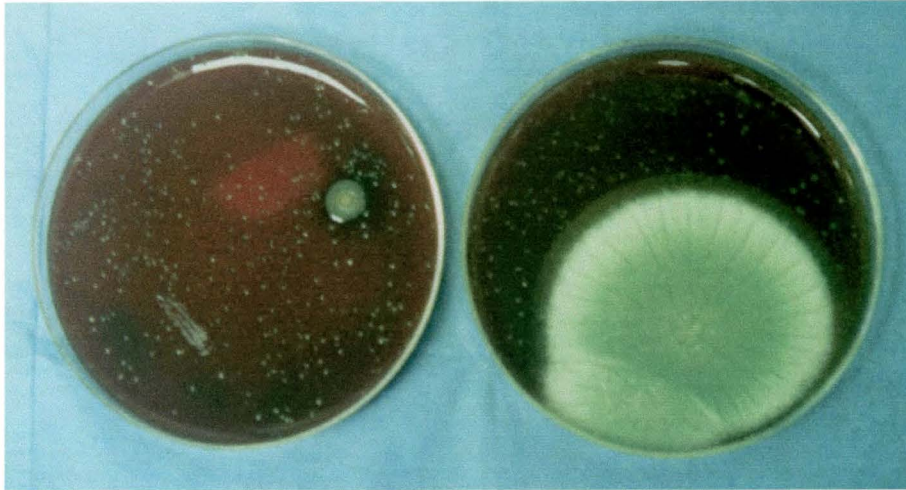


Figure 3. *C. fetus* subsp. *venerealis*. The sample on the left was treated with Weybridge TEM, 4 hours transport, and Skirrow's Agar. The sample on the right was treated with Weybridge TEM, 4 hours transport, and Greenbriar Plus Agar. Accurate diagnosis may be possible for each culture despite the presence of *Pseudomonas* and mold contaminants, respectively.

**Table 1.** Frequency of *Campylobacter fetus* subsp. *venerealis* growth in categories within variables according to growth scores.

Variable	Category	n	Score				
			0	1	2	3	4
Transport Enrichment Media							
	Cary and Blair <sup>a</sup>	71	53	2	3	0	13
	Saline Solution <sup>a</sup>	72	59	1	1	1	10
	Weybridge <sup>b</sup>	67	22	9	7	5	24
Transport Time							
	4 Hours <sup>b</sup>	103	52	5	10	5	31
	24 Hours <sup>a</sup>	107	82	7	1	1	16
Incubation of TEM							
	No Incubation <sup>b</sup>	102	56	1	3	3	39
	Overnight Incubation <sup>a</sup>	108	78	11	8	3	8
Growth Media							
	Blood Agar <sup>a</sup>	70	50	5	2	1	12
	Greenbriar Plus Agar <sup>a</sup>	69	46	3	4	2	14
	Skirrow's Agar <sup>a</sup>	71	38	4	5	3	21

Growth scores: 0 = 0 cfu; 1 = 1-10 cfu; 2 = 11-50 cfu; 3 = 51-100 cfu; 4 = >100 cfu  
(cfu = colony forming units)

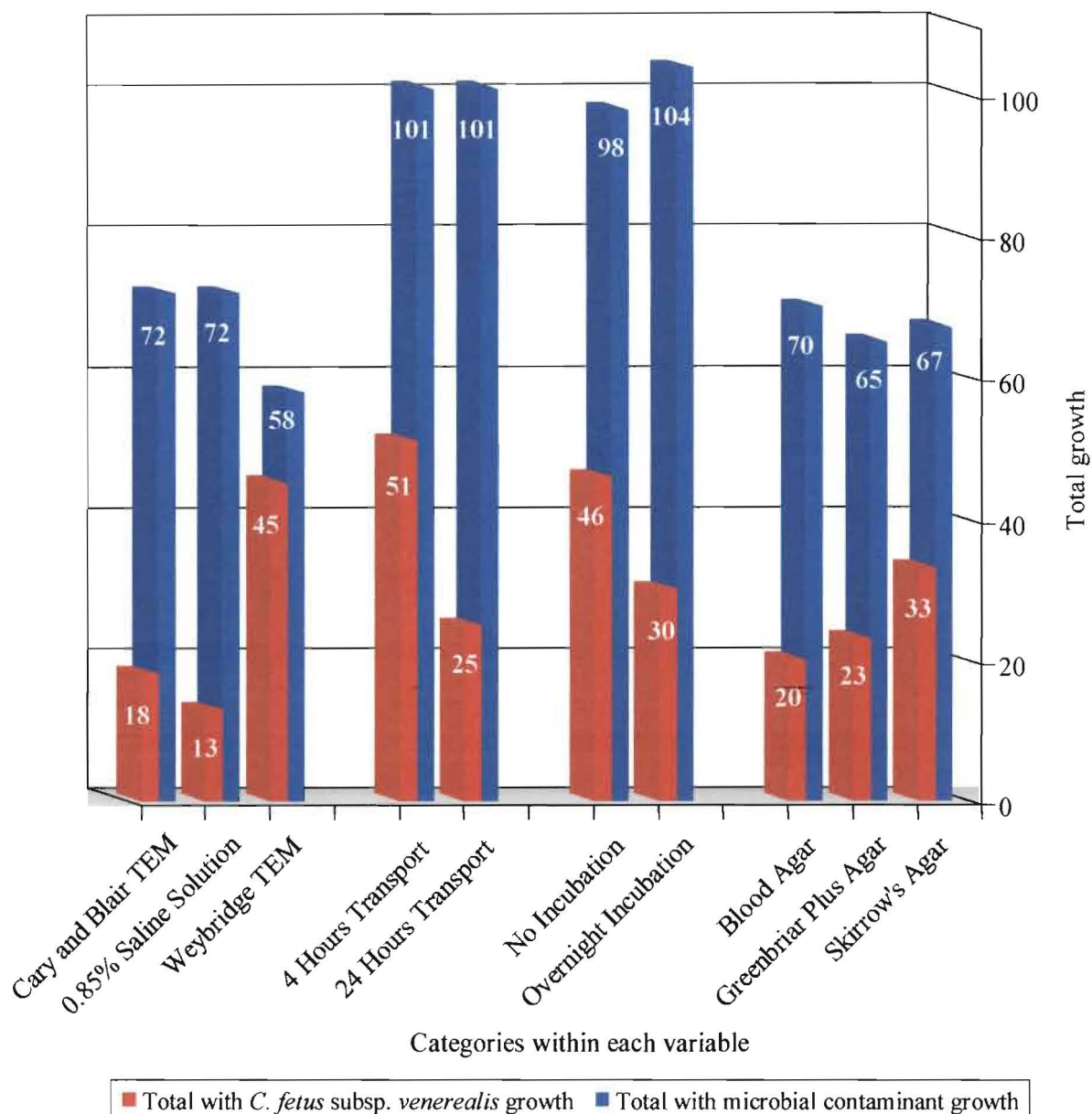
Differences in *C. fetus* subsp. *venerealis* growth are present among categories within each of the variables ( $P < 0.05$ ) as denoted by superscripts. The three variables containing categories denoted by lowercase *b*'s affected *C. fetus* subsp. *venerealis* growth.

**Table 2.** Frequency of microbial contaminants growth in categories within variables according to growth scores.

Variable	Category	n	Score				
			0	1	2	3	4
Transport Enrichment Media							
	Cary and Blair <sup>a</sup>	72	0	8	5	5	54
	Saline Solution <sup>a</sup>	72	0	6	3	5	58
	Weybridge <sup>b</sup>	71	13	38	12	4	4
Transport Time							
	4 Hours <sup>a</sup>	107	6	30	10	7	54
	24 Hours <sup>a</sup>	108	7	22	10	7	62
Incubation of TEM							
	No Incubation <sup>b</sup>	107	9	36	8	4	50
	Overnight Incubation <sup>a</sup>	108	4	16	12	10	66
Growth Media							
	Blood Agar <sup>a</sup>	72	2	15	2	1	52
	Greenbriar Plus Agar <sup>a</sup>	71	6	12	5	4	44
	Skirrow's Agab <sup>b</sup>	72	5	25	13	9	20

Growth scores: 0 = 0% growth; 1 = 1-25% growth; 2 = 26-50% growth; 3 = 51-75% growth; 4 = 76-100% growth (percentage of growth media covered by contaminant growth)

Differences in microbial contaminant growth are present among categories within each of the variables ( $P < 0.05$ ) as denoted by superscripts. The three variables containing categories denoted by lowercase b's affected microbial contaminant growth.



**Figure 4.** Frequency of *C. fetus* subsp. *venerealis* and microbial growth present as a result of each variable. The category with each variable that yields that most *C. fetus* subsp. *venerealis* is preferred. The category within each variable that yields that fewest total microbial contaminant growth is preferred.

0.85% saline solution ( $P < 0.0001$ ). Transport time of 4 hours rather than 24 hours are superior ( $P < 0.0001$ ). Benefits are associated with avoiding overnight TEM incubation ( $P = 0.0002$ ). Significant differences are not identified for growth media; however, Skirrow's Agar yields slightly better growth than either blood agar or Greenbriar Plus Agar.

The Mann-Whitney U test and Kruskal-Wallis test indicate that contaminant growth is also significantly influenced by three of the four variables. Differences associated with TEM indicate Weybridge TEM inhibits contaminant growth more effectively than either Cary and Blair TEM or 0.85% saline solution ( $P < 0.0001$ ). Transport times of 4 and 24 hours do not significantly influence contaminant growth. Abstaining from overnight incubation of TEM is preferred for reduction of contaminant growth ( $P = 0.0032$ ). Skirrow's Agar is preferred to both blood agar and Greenbriar Plus Agar ( $P < 0.0001$ ).

## **Discussion**

These results suggest that the diagnosis of campylobacteriosis is enhanced when preputial smegma samples arrive at the diagnostic laboratory within 4 hours of collection using Weybridge TEM and when direct inoculation of Skirrow's Agar occurs the day samples arrive. Adherence to these guidelines will facilitate accurate diagnosis of bovine genital campylobacteriosis in bulls, thereby reducing the potential for seminal transmission of *C. fetus* subsp. *venerealis* and subsequent occurrence of infertility, early embryonic death, and abortion.

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## **Sources and manufacturers**

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- c. Difco Laboratories, Inc., Detroit, MI.
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